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(54) Process for Preparing Purified Immune Globulin (IgG)

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cess for the preparation of purified immune globulin (IgG) which comprises (a) contacting an aqueous animal, preferably human, plasma fraction containing IgG with a first anionic exchanger to produce an IgG-rich fraction and then (b) contacting said IgG-rich fraction with a second different anionic exchanger to produce a purified IgG-rich fraction.

The invention as claimed herein is furthermore an improved process for the preparation of purified immune globulin (IgG) which comprises (a) passing an aqueous animal, preferably human, plasma fraction containing IgG through a first chromatographic separation column containing a first anionic exchanger to produce an IgG-rich fraction and then (b) passing said IgG-rich fraction through a second chromatographic separation column containing a second different anionic exchanger to produce a purified IgG-rich fraction.

The first anionic exchanger, which removes a substantial proportion of unwanted proteins, may ben an agarose cross-linked support anionic exchanger, such as DEAE-Sepharose* CL6B or DEAE-Biogel*. The DEAE-Sepharose* CL6B may be used in conjunction with a buffer which may be from about 0.06M to about 0.08M sodium acetate, preferably about 0.07M sodium acetate, at a pH within the range of from about 4.8 to about 5.6, preferably at a pH of about 5.2. The DEAE-Biogel* anionic exchanger may be used in conjunction with a buffer which may be from about 0.015 to about 0.025M sodium acetate, preferably about 0.02M sodium acetate, at a pH within the range of from about 5.6 to about 6.4, preferably at a pH of about 6.0.

The second anionic exchanger which is used to remove the remaining minor amounts of unwanted proteins from the IgG-rich fraction may be any appropriate anionic exchanger known to be effective in adsorbing proteins from aqueous solution. Thus the second anionic exchanger may be, for ex-

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plasma to the column, an IgG-rich solution is obtained in the form of an eluate by elution of the column with the equilibrating buffer, i.e., the same sodium acetate buffer which is preferably 0.07M sodium acetate at a pH of 5.2.

Alternatively, if DEAE-Biogel* is to be used as the anionic exchanger in the first anion exchange column, the buffer to be used for initially conditioning or equilibrating the plasma, for pre-equilibrating the column and for eluting the column to provide an IgG-rich solution in the form of an eluate maybe within the range of from about 0.015M to about 0.025M, preferably be 0.02M sodium acetate at a pH of from about 5.6 to about 6.4, preferably about 6.0.

3. The IgG-rich dilute solution obtained as eluate from the first anionic exchange column is concentrated by ultrafiltration to about 0.5g% to 1.0g% protein concentration followed by diafiltration against 2 to 3 volumes of the eluting buffer which is to be used in the second anion exchange column.

4. The concentrated and diafiltered IgG-rich solution is then applied to an equilibrated second anion exchange column for further purification. If DEAE-Sepharose* CL6B has been used in the first anion exchange column, the second anion exchange column preferably contains either DEAE-Biogel* or DEAE-Sephadex* A-50 as the second anionic exchanger. When DEAE-Biogel* is used in the second exchange column, the equilibrating and eluting buffer may be from about 0.015M to about 0.025M sodium acetate, preferably 0.02M sodium acetate, at a pH of about 5.6 to about 6.4, preferably at about 6.0. When DEAE-Sephadex* A-50 is used in the second anion exchange column, the equilibrating and eluting buffer may be from about 0.02M to about 0.03M potassium phosphate, preferably 0.025M potassium phosphate, at

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a pH of about 7.1 to about 7.9, preferably at about 7.5.

Following application of the IgG-rich solution to the second anion exchange column, elution may be performed with the appropriate equilibrating buffer. The eluate provides a dilute solution of pure IgG. This dilute solution of IgG may be stabilized by the addition of sodium chloride, for example to a concentration of 0.15M sodium chloride, and glycine, for example to a concentration of 0.1M glycine. The stabilized solution thus obtained may then be concentrated by ultrafiltration.

When DEAE-Biogel is used as the anionic exchanger in the first anion exchange column, it is preferable to use either DEAE-Sephadex A-50 or DEAE-Sepharose CL6B as the anionic exchanger in the second anion exchange purification column. The procedure for application of the crude IgG solution to the column, elution of the column, and concentration of the dilute pure IgG, which may or may not be stabilized, is similar to the procedure outlined in paragraph 4 above. The equilibrating and eluting buffer to be used with DEAE-Sephadex A-50 in the second anion exchange column may be from about 0.02M to about 0.03M potassium phosphate, preferably 0.025M potassium phosphate, at a pH of about 7.1 to about 7.9, preferably at about 7.5. Alternatively, the equilibrating and eluting buffer to be used with DEAE-Sepharose CL6B in the second anion exchange column may be from about 0.025M to about 0.035M sodium acetate, preferably 0.03M sodium acetate, at a pH of from about 4.8 to about 5.6, preferably at about 5.2.

5. The concentrated pure IgG solution obtained as the eluate from the second anion exchange column may be further stabilized by the addition of mannitol within the range of from about 5% to about 10%, preferably about 7%,

and adjustment of the solution to a pH within the range of from about 6.0 to about 6.5, preferably about 6.2. The concentration of IgG in such a solution is from about 4g% to 10g% and the stabilized solution contains 0.15M sodium chloride, 0.1M glycine and from about 5 to about 10%, preferably about 7%, of mannitol as stabilizers. This stabilized IgG solution is then sterile filtered and is thereafter ready for vialing. In certain cases, it may be stored as such ready for use and then used in this form. If desired, the vialled solution may be freeze-dried in order to provide additional stabilization for the IgG product in the form of a solid stabilized composition.

The process of this invention will be described further with particular reference to the manufacture of human serum immune globulin (HSIG) according to the following procedure:

Materials. Membrane filters and ultrafilter membranes were purchased from Millipore Ltd. (Toronto, Canada). DEAE-Sepharose CL6B and DEAE-Sephadex A-50 were purchased from Pharmacia (Canada) Ltd. (Montreal). DEAE-Biogel was purchased from Biorad Laboratories, Richmond, California. All other chemicals were of ACS reagent grade.

EXAMPLE

About 20 litres of animal plasma, such as human plasma, is thawed and asceptically pooled. The cryoprecipitate which is thus formed is then removed by centrifugation at 5200 rpm for 15 minutes to provide a plasma supernatant liquid. This supernatant liquid is treated with DEAE-Sephadex A-50 at an amount of 1 gm/litre by adding the DEAE-Sephadex A-50 to the liquid, mixing and then allowing the mixture to settle for about 30 minutes. The mixture

is filtered to remove the DEAE-Sephadex A-50 which contains unwanted complex products adsorbed thereto. The filtrate is a plasma solution containing IgG to be used as starting material.

The ionic strength of this plasma filtrate is reduced by diafiltration against 4 volumes of pyrogen-free water for injection (WFI). The diafiltered plasma is then sterilized by serial filtration down to 0.20μ Pall filter into a sterile 50 litre polypropylene carboy and there is thus obtained the sterile plasma filtrate containing IgG which is ready for the first anionic exchange fractionation step on an anion exchange column.

The first anion exchange column contains a DEAE-Sepharose CL6B anion exchanger (or a similar kind of an agarose anion exchanger for this first stage of purification). 15 Litres of DEAE-Sepharose CL6B is prepared by an initial wash with a 0.1M sodium acetate buffer at pH 6.0, followed by sterilization by autoclaving at 121°C., 15 psi for 30 min. The sterilized DEAE-Sepharose CL6B is packed into a 15 cm x 370 cm plastic column by the procedure recommended by the supplier of the anion exchanger. The packed column is then equilibrated with a buffer which is a 0.07M sodium acetate buffer at pH 5.2, the buffer passing at a flow rate of 25 litres/hour and at a temperature of 4° to 8°C. This equilibration procedure requires approximately 100 litres of buffer.

An aliquot of 10 litres of the sterile plasma filtrate containing IgG, prepared as above, is applied to the equilibrated column of DEAE-Sepharose CL6B via sterilized polypropylene tubing and a metering pump to provide a flow rate of 25 litres/hour of filtrate to the column. The elution of protein is monitored with a continuous flow U.V.

monitor using a 280 nm wavelength. The eluting protein, which is an IgG-rich fraction and primarily IgG, is collected in a 50 litre sterilized polypropylene container and this eluate is hereafter referred to as DC1. Following complete application of the 10 litres of plasma filtrate to the column, the elution of IgG from the column is continued using 0.07M sodium acetate buffer at pH 5.2, thus providing more DC1 eluate. The plasma proteins not eluted from the column with this 0.07M sodium acetate buffer at pH 5.2 (which include albumin and the other α-globulins) are subsequently eluted from the DEAE-Sepharose CL6B column with sodium acetate buffers of higher ionic strength and lower pH. Following elution with the third buffer the column of DEAE-Sepharose CL6B is re-equilibrated with 0.07M sodium acetate buffer at pH 5.2, in preparation for application of another 10 litres batch of sterile plasma filtrate. The DEAE-Sepharose CL6B column may be re-used in this way for numerous fractionation cycles before fresh DEAE-Sepharose CL6B must be charged to the column.

The IgG-rich eluate, DC1, is concentrated to about 20 litres by ultrafiltration with a Millipore Pellicon U.F. system, followed by diafiltration on the same system against 45 litres of 0.025M potassium phosphate buffer at pH 7.5. This IgG-rich solution is then ready for the second anion exchange column to achieve fractionation on DEAE-Sephadex A-50 as the second anionic exchanger.

The DEAE-Sephadex A-50 is prepared for column fractionation by the following technique. An aliquot of 750 g of DEAE-Sephadex A-50 is transferred to 20 litres of 0.1M potassium phosphate buffer at pH 7.5, incubated for 1 hour and then sterilized by autoclaving at 121°C., 15 psi for 30 min. The sterilized DEAE-Sephadex A-50 slurry is trans-

ferred to a 15 cm x 370 cm sectional column and packed according to the instructions of the supplier, Pharmacia Fine Chemicals. During packing the DEAE-Sephadex A-50 is depyrogenated in the column by washing with 8 litres of the following series of solutions: 0.5M hydrochloric acid, WFI, 0.05M sodium hydroxide, WFI and 0.25M potassium phosphate buffer at pH 7.5. The packed column of DEAE-Sephadex A-50 is finally equilibrated with 0.025M potassium phosphate buffer at pH 7.5 at a flow rate of about 15 litres/hour. This equilibration requires about 150 litres of buffer. There is thus prepared the second anion exchange column containing DEAE-Sephadex A-50 for purification of the IgG-rich solution.

The IgG-rich solutions, prepared as above, from two 10 litre plasma fractionations on the first anion exchange column are pooled and are applied as one sample to the equilibrated second anion exchange column containing DEAE-Sephadex A-50. Following the complete application of the IgG-rich solution to the column, the eluate obtained from the column contains pure IgG and is collected in a sterilized 50 litre polypropylene container. Elution of the column is continued by use of 0.025M potassium phosphate buffer at pH 7.5. The dilute solution of pure IgG thus obtained (about 40 litres) is stabilized by the addition of sufficient glycine and sodium chloride to provide a concentration of 0.1M glycine and 0.15M sodium chloride in the dilute solution of IgG. The dilute solution of IgG thus stabilized (about 44 litres) is subsequently concentrated to about one litre by ultrafiltration with a Millipore Pellicon U.F. System using a PTGC 000 05 cassette membrane (NMWL of 10,000/5ft.^{2}) (Millipore Ontario Canada). Finally, the concentrated solution of IgG is further stabilized by the addition of 7% mannitol.

The pure concentrated IgG, hereafter referred to as HSIG i.v., was clarified by centrifugation at 5200 rpm for 30 min. at 4°C. and was then sterile filtered with a 0.2μ membrane filter in preparation for vialling.

The HSIG i.v. prepared as described above was shown to be pure IgG following examination by immunoelectrophoresis and by double diffusion against anti-human whole serum. HSIG i.v. contained less than 2% aggregated IgG and less than 1% fragmented IgG. The anti-complementary activity assays of 5g% to 8g% solutions of HSIG i.v. yielded titres of 1/8 or less indicating suitability for intravenous administration. Several lots of HSIG i.v. have been produced and the quality of control results indicate they meet the pyrogen-free, safety, sterility, hepatitis B surface antigen-free, and purity requirements of the Bureau of Biologics, Health Protection Branch, Health and Welfare, Canada.

It will be appreciated, by one skilled in the art, that the DEAE-Sepharose CL6B or DEAE-Biogel used as the anionic exchanger in the first anion exchange column may be replaced by any other agarose anionic exchanger. Likewise, the DEAE-Sepharose CL6B or DEAE-Sephadex A-50 or DEAE-Biogel used as the anionic exchanger in the second anion exchange column may be replaced by any other suitable anionic exchanger having similar anion exchange properties in being effective in the adsorption of unwanted proteins. It will be understood that the first anionic exchanger removes the bulk of the unwanted proteins, possibly of the order of about 85% or about 90%, from the plasma fraction used as starting material to provide an IgG-rich solution. The second anionic exchanger is effective in removing the residual unwanted proteins from that solution thereby providing a solution containing pure IgG. The second anionic

exchanger may thus, for example, be DEAE-Sepharose CL6B, DEAE-Sephadex A-50, DEAE-Biogel, DEAE-Trisacryl, a DEAE-cellulose (granular cellulose support) or DEAE-Sephocel (a beaded form cellulose).

It is preferred that the anionic exchanger to be used in the first column is DEAE-Sepharose CL6B and the anionic exchanger to be used in the second column is DEAE-Sephadex A-50.

When the anionic exchanger to be used in the first column is DEAE-Sepharose CL6B, the buffer used for equilibrating the column and for elution may be from about 0.06M to about 0.08M sodium acetate, preferably 0.07M sodium acetate, at a pH within the range of from about 4.8 to about 5.6, preferably at a pH of about 5.2. Under these conditions, the anionic exchanger to be used in the second column may be either DEAE-Biogel with a buffer from about 0.015M to about 0.025M sodium acetate, preferably 0.02M sodium acetate, at a pH from about 5.6 to about 6.4, preferably at pH 6.0, or DEAE-Sephadex A-50 with a buffer from about 0.02M to about 0.03M potassium phosphate, preferably 0.025M potassium phospate, at a pH of from about 7.1 to about 7.9, preferably a pH of about 7.5.

When the anionic exchanger to be used in the first column is DEAE-Biogel, the buffer used for equilibrating the column and for elution may be from about 0.015M to about 0.025M sodium acetate, preferably 0.02M sodium acetate at a pH of from about 5.6 to about 6.4, preferably at pH about 6.0. Under these conditions, the anionic exchanger to be used in the second column may be either DEAE-Sepharose CL6B with a buffer from about 0.025M to about 0.035M sodium acetate, preferably 0.03M sodium acetate at a pH from about 4.8 to about 5.6, preferably a pH of about 5.2 or DEAE-Sephadex

A-50 with a buffer from about 0.02M to about 0.03M potassium phosphate, preferably about 0.025M potassium phosphate, at a pH from about 7.1 to about 7.9, preferably at a pH of about 7.5.

When the anionic exchanger to be used in the second column is DEAE-Trisacryl*, a suitable buffer may be 0.025M potassium phosphate at a pH of about 7.5.

When the anionic exchanger to be used in the second column is DEAE-cellulose (granular cellulose support) or DEAE-Sephacel* (a beaded form cellulose), a suitable buffer may be 0.025M potassium phosphate at a pH of about 7.5 or 0.03M sodium acetate at a pH of about 5.2 or 0.02M sodium acetate at a pH of about 6.0.

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The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:-

1. An improved process for the preparation of purified immune globulin (IgG) which comprises (a) contacting an aqueous animal plasma fraction containing IgG with a first anionic exchanger to produce an IgG-rich fraction and then (b) contacting said IgG-rich fraction with a second different anionic exchanger to produce a purified IgG-rich fraction.

2. An improved process for the preparation of purified immune globulin (IgG) which comprises (a) passing an aqueous animal plasma fraction containing IgG through a first chromatographic separation column containing a first anionic exchanger to produce an IgG-rich fraction and then (b) passing said IgG-rich fraction through a second chromatographic separation column containing a second different anionic exchanger to produce a purified IgG-rich fraction.

3. The process as in claim 2 wherein the first anionic exchanger is an agarose cross-linked support anionic exchanger.

4. The process as in claim 2 wherein the first anionic exchanger is DEAE-Sepharose CL6B or DEAE-Biogel.

5. The process as in claim 2 wherein the first anionic exchanger is DEAE-Sepharose CL6B and the IgG-rich fraction is obtained therefrom as an eluate by use of a buffer which contains from about 0.06M to about 0.08M sodium acetate at a pH within the range of from 4.8 to 5.6.

6. The process as in claim 2 wherein the first anionic exchanger is DEAE-Sepharose CL6B and the IgG-rich fraction is obtained therefrom as an eluate by use of a buffer which is 0.07M sodium acetate at a pH of 5.2.

7. The process as in claim 2 wherein the first anionic exchanger is DEAE-Biogel and the IgG-rich fraction is obtained therefrom as an eluate by use of a buffer which contains from about 0.015M to about 0.025M sodium acetate at a pH within the range of from 5.6 to 6.4.

8. The process as in claim 2 wherein the first anionic exchanger is DEAE-Biogel and the IgG-rich fraction is obtained therefrom as an eluate by use of a buffer which is 0.02M sodium acetate at a pH of 6.0.

9. The process as in claim 2 wherein the second anionic exchanger is selected from the group consisting of DEAE-Sepharose CL6B, DEAE-Biogel and DEAE-Sephadex A-50.

10. The process as in claim 2 wherein the second anionic exchanger is DEAE-Sepharose CL6B and the purified IgG-rich fraction is obtained therefrom as an eluate by use of a buffer which contains from about 0.025M to about 0.035M sodium acetate at a pH within the range of 4.8 to 5.6.

11. The process as in claim 2 wherein the second anionic exchanger is DEAE-Sepharose CL6B and the purified IgG-rich fraction is obtained therefrom as an eluate by use of a buffer which is 0.03M sodium acetate at a pH of 5.2.

12. The process as in claim 2 wherein the second anionic exchanger is DEAE-Biogel and the purified IgG-rich fraction is obtained therefrom as an eluate by use of a buffer which contains from about 0.015M to about 0.025M sodium acetate at a pH within the range of 5.6 to 6.4.

13. The process as in claim 2 wherein the second anionic exchanger is DEAE-Biogel and the purified IgG-rich fraction is obtained therefrom as an eluate by use of a buf-

fer which is 0.02M sodium acetate at a pH of 6.0.

14. The process as in claim 2 wherein the second anionic exchanger is DEAE-Sephadex A-50 and the purified IgG-rich fraction is obtained therefrom as an eluate by use of a buffer which contains from about 0.02M to about 0.03M potassium phosphate at a pH within the range of 7.1 to 7.9.

15. The process as in claim 2 wherein the second anionic exchanger is DEAE-Sephadex A-50 and the purified IgG-rich fraction is obtained therefrom as an eluate by use of a buffer which is 0.025M potassium phosphate at a pH of 7.5.

16. The process as in claim 2 wherein the first anionic exchanger is DEAE-Sepharose CL6B, the IgG-rich fraction being obtained therefrom as an eluate by use of a buffer which contains from about 0.06M to about 0.08M sodium acetate at a pH within the range of 4.8 to 5.6, and the second anionic exchanger is either DEAE-Biogel, the purified IgG-rich fraction being obtained therefrom as an eluate by use of a buffer which contains from about 0.015M to about 0.025M sodium acetate at a pH within the range of 5.6 to 6.4, or DEAE-Sephadex A-50, the purified IgG-rich fraction being obtained therefrom as an eluate by use of a buffer which contains from about 0.02M to about 0.03M potassium phosphate at a pH within the range of 7.1 to 7.9.

17. The process as in claim 2 wherein the first anionic exchanger is DEAE-Sepharose CL6B, the IgG-rich fraction being obtained therefrom as an eluate by use of a buffer which is 0.07M sodium acetate at a pH of 5.2, and the second anionic exchanger is either DEAE-Biogel, the purified IgG-rich fraction being obtained therefrom as an eluate by use of a buffer which is 0.02M sodium acetate at pH 6.0, or DEAE-

Sephadex A-50, the purified IgG-rich fraction being obtained therefrom as an eluate by use of a buffer which is 0.025M potassium phosphate at a pH of 7.5.

18. The process as in claim 2 wherein the first anionic exchanger is DEAE-Biogel, the IgG-rich fraction being obtained therefrom as an eluate by use of a buffer which contains from about 0.015M to about 0.025M sodium acetate within a pH range of 5.6 to 6.4, and the second anionic exchanger is either DEAE-Sepharose CL6B, the purified IgG-rich fraction being obtained therefrom as an eluate by use of a buffer which contains from about 0.025M to about 0.035M sodium acetate within a pH range of 4.8, to 5.6, or DEAE-Sephadex A-50, the purified IgG-rich fraction being obtained as an eluate by use of a buffer which contains from about 0.02M to about 0.03M potassium phosphate within a pH range of 7.1 to 7.9.

19. The process as in claim 2 wherein the first anionic exchanger is DEAE-Biogel, the IgG-rich fraction being obtained therefrom as an eluate by use of a buffer which is 0.02M sodium acetate at a pH of 6.0, and the second anionic exchanger is either DEAE-Sepharose CL6B, the purified IgG-rich fraction being obtained therefrom as an eluate by use of a buffer which is 0.03M sodium acetate at a pH of 5.2, or DEAE-Sephadex A-50, the purified IgG-rich fraction being obtained as an eluate by use of a buffer which is 0.025M potassium phosphate at a pH of 7.5.

20. The process as in claim 2 wherein the second anionic exchanger is selected from the group consisting of DEAE-Trisacryl, a DEAE-cellulose and DEAE-Sephocel.

21. The process as in claim 2 wherein the purified IgG-rich fraction is stabilized by the addition of sodium

chloride and glycine.

22. The process as in claim 2 wherein the purified IgG-rich fraction is stabilized by the addition of sodium chloride, glycine and mannitol.

23. The process as in claim 2 wherein the purified IgG-rich fraction is stabilized and concentrated to produce a product containing pure IgG within the range of from about 4g% to about 10g% containing 0.15M sodium chloride and 0.1M glycine.

24. The process as in claim 2 wherein the purified IgG-rich fraction is stabilized and concentrated to produce a product containing pure IgG within the range of from about 4g% to about 10g% containing 0.15M sodium chloride, 0.1M glycine and from about 5% to about 10% mannitol.

25. The process as in claim 2 wherein the aqueous animal plasma fraction used as starting material is an aqueous human plasma fraction.

26. The process as in claim 25 wherein the human plasma fraction is either normal human plasma or human plasma containing a high-titer of Rh factor antibodies, rabies antibodies, tetanus antibodies or zoster antibodies.

27. An improved process for the preparation of purified immune globulin (IgG) which comprises (a) passing an aqueous animal plasma fraction containing IgG through a first chromatographic separation column containing a first anionic exchanger to produce an IgG-rich fraction, (b) subjecting said IgG-rich fraction to concentration and equilibration for a second chromatographic separation column and then (c) passing said IgG-rich fraction through a second chromatographic sepa-

ration column containing a second different anionic exchanger to produce a purified IgG-rich fraction.

28. An improved process for the preparation of purified immune globulin (IgG) which comprises (a) passing an aqueous animal plasma fraction containing IgG through a first chromatographic separation column containing a first anionic exchanger and eluting said column with an aqueous buffer solution to provide an IgG-rich fraction, (b) subjecting said IgG-rich fraction to concentration and diafiltration to provide an equilibrated solution and then (c) passing said equilibrated solution through a second chromatographic separation column containing a second different anionic exchanger and eluting said second column to provide a purified IgG-rich fraction.

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